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Studies of RBC Preservation In-Vivo in a Rabbit Model

Final Report

Irma O. Szymanski, M.D.

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During blood storage at 4C, a progressively larger fraction of RBC become nonviable. Upon transfusion, the nonviable RBC are cleared extravascularly, but the exact site of their removal is not known. The number of nonviable RBC in a unit of blood can be determined only by transfusion studies in vivo. Both single and double label methods have been used to quantitate the number of nonviable RBC, although there is controversy about the accuracy of the single label method. It is not known which changes in stored RBC cause their rapid removal from circulation. The third component of complement accumulates on the RBC membrane during storage at 4C. It is possible that the C3 bound to stored RBC plays a role in the storage lesion by facilitating the rapid removal of the nonviable RBC. We conducted transfusion studies in rabbits to evaluate these issues in RBC preservation. Since some of them are difficult to evaluate in human subjects, the use of rabbit model is valuable.

Analysis of the survival data showed that normal, fresh autologous rabbit RBC were eliminated at a single exponential rate having a t 1/2 of 12.7 days as determined by radioactive

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chromium technic. In contrast, the stored RBC were eliminated in two phases: during the
first 24 hours the nonviable RBC were removed rapidly whereas after the 24 hours the rate
of removal approached that of the fresh RBC. Even in the first 24 hours the rate of removal
of nonviable stored RBC had more than one component. This is shown by analysis of the survival
curves during the first one hour: the survival curves had y-intercepts below 100%, the
mean value being about 92%. This indicates that some of the nonviable RBC were eliminated
rapidly during the transfusion. Thus, utilization of the single label to measure preser-
vation injury would be inaccurate. Our results also show that stored RBC surviving at 24
hours after transfusion have a potential for normal long term survival.

Our data showed that senescent rabbit RBC were destroyed in bone marrow and spleen.
We were unable to demonstrate any RBC destruction in liver. In contrast, over 75% of the
stored nonviable RBC were destroyed in bone marrow, 16 - 21% in the liver and the remainder
in the spleen. The liver removed the nonviable RBC rapidly whereas the destruction in the
spleen and bone marrow took place over a longer period of time. It is possible that the
phagocytosis in the liver proceeded by a different mechanism than in the spleen and bone
marrow.

RBC stored in C3 depleted CPD plasma failed to accumulate C3 on their membranes. In
some cases the RBC survival was good, whereas in other cases the survival was very poor.
we postulate reactive hemolysis as a cause of poor survival in these cases. It appears that
accumulation of C3 to RBC membrane during storage is not the only component of the preser-
vation injury.

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SUMMARY

During blood storage at 4C, a progressively larger fraction of RBC become nonviable. Upon transfusion, the nonviable RBC are cleared extravascularly, but the exact site of their removal is not known. The number of nonviable RBC in a unit of blood can be determined only by transfusion studies in vivo. Both single and double label methods have been used to quantitate the number of nonviable RBC, although there is controversy about the accuracy of the single label method. It is not known which changes in stored RBC cause their rapid removal from the circulation. The third component of complement accumulates on the RBC membrane during storage at 4C. It is possible that the C3 bound to stored RBC plays a role in the storage lesion by facilitating the rapid removal of the nonviable RBC. We conducted transfusion studies in rabbits to evaluate these issues in RBC preservation. Since some of them are difficult to evaluate in human subjects, the use of rabbit model is valuable.

Analysis of the survival data showed that normal, fresh autologous rabbit RBC were eliminated at a single exponential rate having a t 1/2 of 12.7 days as determined by radioactive chromium technic. In contrast, the stored RBC were eliminated in two phases: during the first 24 hours the nonviable RBC were removed rapidly whereas after the 24 hour the rate of removal approached that of the fresh RBC. Even in the first 24 hours the rate of removal of nonviable stored RBC had more than one component. This is shown by analysis of the survival curves during the first one hour: the survival curves had y-intercepts below 100%, the mean value being about 92%. This indicates that some of the nonviable RBC were eliminated rapidly during the transfusion. Thus, utilization of the single label to measure preservation injury would be inaccurate. Our results also show that stored RBC surviving at 24 hours after transfusion have a potential for normal long term survival.

Our data showed that senescent rabbit RBC were destroyed in bone marrow and spleen. We were unable to demonstrate any RBC destruction in liver. In contrast, over 75 % of the stored nonviable RBC were destroyed in bone marrow, 16 to 21% in the liver and the remainder in the spleen. The liver removed the nonviable RBC rapidly whereas the destruction in the spleen and bone marrow took place over a longer period of time. It is possible that the phagocytosis in the liver proceeded by a different mechanism than in the spleen and bone marrow.

RBC stored in C3 depleted CPD plasma failed to accumulate C3 on their membranes. In some cases the RBC survival was good, whereas in other cases the survival was very poor. We postulate reactive hemolysis as a cause of poor survival in these cases. It appears that accumulation of C3 to RBC membrane during storage is not the only component of the preservation injury.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Councel (DHEW Publication No. (NIH) 78-23, Revised 1978).

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STATEMENT OF THE PROBLEM:

During blood storage at 4C, a fraction of the red blood cells (RBC) becomes nonviable. The larger this fraction, the poorer the quality of RBC preservation. Unfortunately, the fraction of viable RBC in stored blood can be measured only by survival studies in vivo (1). Human studies are difficult due to limited numbers of willing subjects. Thus, we have investigated whether a rabbit model could be used to evaluate RBC preservation, and whether the data obtained in this model are applicable to the human system.

Recently there has been considerable controversy over the best method to measure the preservation injury, i.e., the percentage of nonviable RBC in the stored unit of blood. Some advocate the use of a single radioactive label (2) to tag the stored transfused RBC. Their 24 hour post-transfusion survival is then measured as a percentage of the survival immediately after transfusion. Although this method is simple, others argue that it is not accurate and that the survival can be measured precisely only by a double label technic (3) in which autologous RBC are also labeled so that the expected 100% recovery of stored RBC in vivo can be calculated. Inaccurate data might result in acceptance of preservation methods that fail to maintain 70% survival of stored RBC (4). Human studies show that the stored non-viable RBC are destroyed within 24 hours in extracellular sites (1). However, it is not known in which organs the nonviable RBC are destroyed. The destruction of antibody-coated RBC in various organs is dependent upon the type of antibody, e.g., RBC coated with complement binding antibodies are preferentially destroyed in liver (5). Thus, knowledge about the site of destruction of nonviable RBC might shed light on the nature of the preservation injury.

Our studies with the rabbit model were carried out to evaluate the technical and biological variables in measuring RBC survival in these animals and to measure the preservation injury of stored rabbit RBC. The rabbit model was also used to evaluate some controversial issues in measuring the preservation injury, e.g., whether double or single label method should be used to measure RBC preservation, and whether 99m-Tc is an adequate RBC label. (The results with 99m-Tc have been reported in the 1985 annual report). We also used the rabbit model to evaluate the rate of removal of the nonviable RBC from the circulation and whether this rate is affected by the preservation technic. The analysis of long-term survival of stored RBC permitted us to determine whether the viable RBC have normal life span.

Utilizing the rabbit model we also determined in which organs the preserved nonviable RBC were destroyed. These experiments involved obtaining quantitative data to determine which organ was most important in clearing nonviable RBC.

Since the third component of complement accumulates on the RBC membrane during liquid storage (6), it was our intent to determine whether this plays a role in the preservation injury. Thus, we removed C3 from the donor rabbits by cobra venom factor treatment (7). Blood collected from these rabbits was stored at 4C, during which time RBC did not become C3-coated. Thus, it was expected that survival studies with these RBC could establish whether C3 coating was involved in the preservation injury.

Results of our studies have already been given in the annual reports of years 1985 and 1986. However, some additional experiments and computer analyses were carried out after July 1986. These analyses are shown in this report.

METHODS

Collection and Storage of Blood from Donor Rabbits:

Prior to blood collection, the donor rabbits were given 1000 units of heparin intravenously. Then the ear surface of each rabbit was cleansed thoroughly with Betadine and isopropyl alcohol. About 20 minutes after heparin injection, approximately 60 ml of whole blood was drawn through the central ear artery using aseptic technic. The blood was dispensed into a plastic blood bag (Fenwal) and an appropriate amount of citrate-phosphate-dextrose (CPD) or CPD-A1 anticoagulant was added (1.4 ml of the anticoagulant for each 10 ml of whole blood). After thorough mixing the blood was stored at 4C until the studies were performed.

Preparation of ADSOL Blood:

The CPD blood was centrifuged at 2000 g for 10 minutes whereafter most of the supernatant plasma was removed and an appropriate amount of ADSOL was added to the packed RBC (2.2 ml ADSOL for each 10 ml of whole blood). The blood was then stored at 4C until needed.

Complement-depletion of Rabbits:

Cobra venom factor (Naja naja, lot #64014 and Naja haje, lot #34025), (Cordis Labs) was given to the rabbits intraperitoneally in a total amount of 250 U/kg, divided into four equal doses (7) . Twenty-four hours after the last injection, a unit of blood was collected from these animals in CPD anticoagulant as described above. The units of blood were stored at 4C until needed in the studies.

51-Cr red cell mass in rabbits :
RBC mass of 149 adult male New Zealand rabbits weighing 3.46 ± 0.40 kg was measured during the last year of the contract. Since these results were not reported before, the data will be given in this report.

To measure RBC mass, three ml of autologous whole blood was collected into a heparinized syringe whereafter 0.14 ml of acid citrate dextrose (ACD, NIH Formula A) was added to each one ml of whole blood. About 3 uCi of 51-Cr sodium chromate was added to the blood sample and incubated at room temperature for at least 30 minutes. No ascorbic acid was added and the RBC were not washed (8). The percent uptake of 51-Cr by RBC was determined. An exact volume of radiolabeled blood was injected intravenously and a blood sample was collected within 15 minutes of the injection to determine the dilution of the labeled RBC in vivo. The RBC mass was calculated as a ratio between the total amount of RBC bound 51-Cr counts given and the counts per ml of packed RBC in the post infusion sample.

Measurement of the Survival of Stored RBC:

Survival of stored RBC was measured either by double 51-Cr technic (8) as described in the annual report of 1986 or with 99m-Tc/51-Cr as described in the annual report of 1985.

Autologous unstored RBC for these studies were labeled with 50 uCi of 51-Cr. In these cases the counts per ml of RBC in the first post-transfusion blood sample, collected within 15 minutes of transfusion were considered to represent 100 percent recovery. Subsequent blood samples were drawn at various times during the initial 48 hours. The counts per ml RBC in the subsequent blood samples were compared to those in the initial blood sample to calculate RBC survival.

Analysis of RBC Survival Curves

Analysis of the survival curves of autologous unstored RBC was done in two phases: short term survivals and long term survivals. The short term survivals consisted of data obtained in 23 rabbits during the first 48 hours after transfusion. The long term survivals consisted of data obtained in 19 rabbits between one and 13 days after transfusion. The short term survival data as well as the long term survival data of all cases were combined. This permitted us to compare the RBC destruction rates of unstored RBC in the two different time periods.

Similar analysis was applied to the survival of stored RBC. The short term survivals of variously stored RBC were analyzed separately. We combined the short term survival data obtained in 21 rabbits who received CPD blood (mean length of storage 19 days). We also combined the short term survival data obtained in 23 rabbits who received ADSOL blood (mean length of storage 31.5 days) and the data obtained in 27 rabbits who received C3 depleted CPD blood (mean length of storage 18.9 days).

The long term survival studies were done in rabbits who received either CPD, CPD-A1, or ADSOL blood. In each case individual regression lines were analyzed. The data were then normalized by assigning 100% value to the y intercept of the regression line. Then all the long term survival data were combined for analytical purposes.

We also determined the relationship between the length of storage and t 1/2 in the individual cases.

Estimation of isotope content in various organs:

Studies with Unstored Blood:

The 23 rabbits receiving unstored autologous RBC and 78 rabbits who received variously stored RBC were sacrificed at different times after transfusion. The liver, spleen and kidneys were removed as soon as possible. In some rabbits the lungs and the total bone marrow of the right femur were also removed. The outer surfaces of the organs were rinsed with 0.9% NaCl, then dried with paper towels and the organs were weighed. Either the whole organ (spleen and the bone marrow removed from the femur) or three or more weighed samples from the organ were counted for radioactivity to determine the mean counts per gram of organ. The

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total amounts of radioactivity in the organs (Tr) were then calculated and expressed as a percent of the total amount of RBC-bound radioactivity given (%oRBC) as shown below:

$$\%oRBC=100*(Tr/TRBC),$$

where TBRC equals the total amount of RBC-bound radioactivity given.

We also determined what percentage of the surviving RBC (%SRBC) the radioactivity in the organ represented using the following formula:

$$\%SRBC=100*(\%oRBC/\%S),$$

where %S equals percent RBC survival.

%oRBC and %SRBC per gram organ weight were also calculated.

We utilized Pearson correlation analysis to determine whether the percent RBC recovery in the organ (%oRBC) depended on organ weight, the percent RBC survival or on the length of time after transfusion. On the basis of the data obtained with unstored RBC, formulas were developed to estimate whether the counts in the organ reflected blood flow or destruction of senescent RBC in that organ.

Studies with Stored Blood:

The quantity of radioactivity in the organs was measured as described above. To determine whether phagocytosis occurred in the organ of question, the normal background (determined in the autologous studies) was subtracted from the total organ radioactivity. The relationship of the quantity of phagocytosis to the percent RBC survival and to the time after transfusion was then determined.

Radioiron Studies:
To estimate what percentage of the total bone marrow was present in a femur, radioiron studies were performed in three rabbits. The animals were given an intravenous injection of 0.5 uCi of 59-Fe as ferrous citrate. Six or seven blood samples were collected between 5 and 265 minutes after the injection. The rabbits were sacrificed between 171 and 265 minutes following the injection and the liver, spleen, lungs, kidneys and bone marrow from both femurs were removed. The organs were counted for 59-Fe radioacitivity as described above for 51-Cr. The rate of disappearance of the radioiron from plasma was determined by plotting the natural logarithm of the counts per ml of plasma as a function of time and determining the parameters of this regression. The average rate of disappearance of 59-Fe was 0.64% per minute as shown in figure 1. On the average, 23% of the injected radioiron (100% equaled the counts at y-intercept) was detected in plasma at the time the animal was sacrificed. On the average, 9.23% of the injected dose was recovered in the removed organs. Although this figure also included some counts due to contamination of the organs with peripheral blood, the contamination was relatively small due to low recovery of the 59-Fe in the blood stream at

PLASMA ^{59}Fe TURNOVER IN THREE RABBITS

$Yt = 98.3 \, e^{-0.0064t}$
$r = -0.983$
$N = 19$
$P = <0.00001$

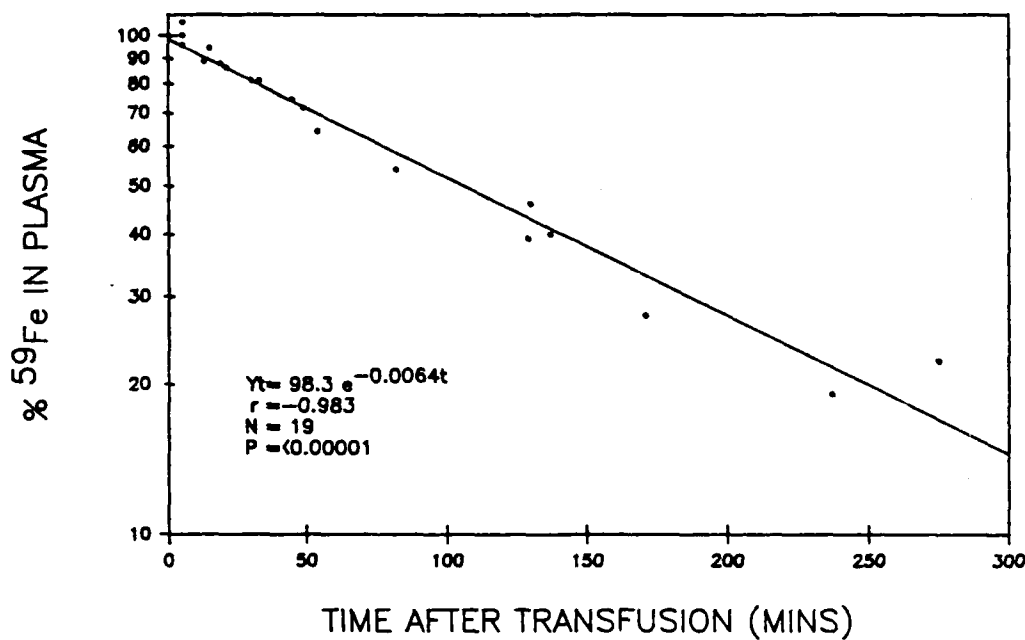X-axis: TIME AFTER TRANSFUSION (MINS)
Y-axis: % ^{59}Fe IN PLASMA

Figure 1.

Disappearance of 59-Fe given to each of three rabbits as ferrous citrate. The individual post infusion values are given as percent of the y-intercept in each case. The average regression line is calculated.

the time of the sacrifice. On the average, 6.94% of the 59-Fe was recovered in the liver indicating iron uptake in that organ. The recovery of 59-Fe in blood and in the organs was subtracted from the total amount given to determine the total counts taken up by the bone marrow. This quantity was 67.7% of the injected radioiron. On the average, 1.92% of the injected dose was recovered in the femoral marrow, which amounts to 2.84% of the bone marrow dose. Therefore, the amount of the radioactivity in the total bone marrow was estimated by multiplying the radioactivity in the femur by 35.2.

RESULTS

Autologous fresh RBC
RBC mass measurements with 51-Cr

51-Cr uptake by autologous RBC was 83.3 ± 3.3% (mean ± S.D.). On the average, rabbit RBC mass was 57.6± 8.56 ml (mean ± S.D.), or 16.7±2.01 ml/kg. The total RBC mass correlated positively with the rabbit weight ($r = 0.615$, $n = 149$, $p < 0.0001$), whereas the RBC mass, expressed as ml/kg, demonstrated a very weak negative correlation with the rabbit weight ($r = -0.200$, $n = 149$, $p = 0.014$) indicating that RBC mass was perhaps better correlated with lean body mass than just the body weight. In the larger rabbits, the fat tissue might have been a proportionally larger fraction of the total body mass so that in these animals the RBC mass per kg of body weight was lower than in the leaner rabbits.

Short-term RBC survival studies of unstored RBC:

RBC survival regression during the first 48 hours after transfusion of unstored autologous RBC is shown in Figure 2. There was a significant negative correlation between the percent survival and time after transfusion ($r = -0.839$, $N = 86$, $p < 0.00001$). The y-intercept of the regression line was at 99.7% survival and the rate of destruction was 0.28 percent per hour, with t 1/2 Cr of 10.3 days.

Long-term RBC survival studies of unstored RBC:

RBC survival regression of the data obtained between 1 and 13 days after transfusion of unstored autologous RBC is shown in Figure 3. The y-intercept of the regression line was at 98.2% survival, the rate of RBC destruction was 5.47% per day and t1/2 Cr was 12.7 days.

Short term RBC survival studies of CPD stored RBC:

RBC survival regression during the first 24 hours after transfusion of CPD stored RBC is shown in Figure 4. The y-intercept was 83.7% and the rate of destruction was 0.97% per hour with t 1/2 of 71.5 hours. There was a significant negative correlation between the time after transfusion and the percent RBC survival ($r = -0.394$, $N = 99$, $p = 0.00004$).

SHORT TERM SURVIVAL OF UNSTORED RABBIT RBC
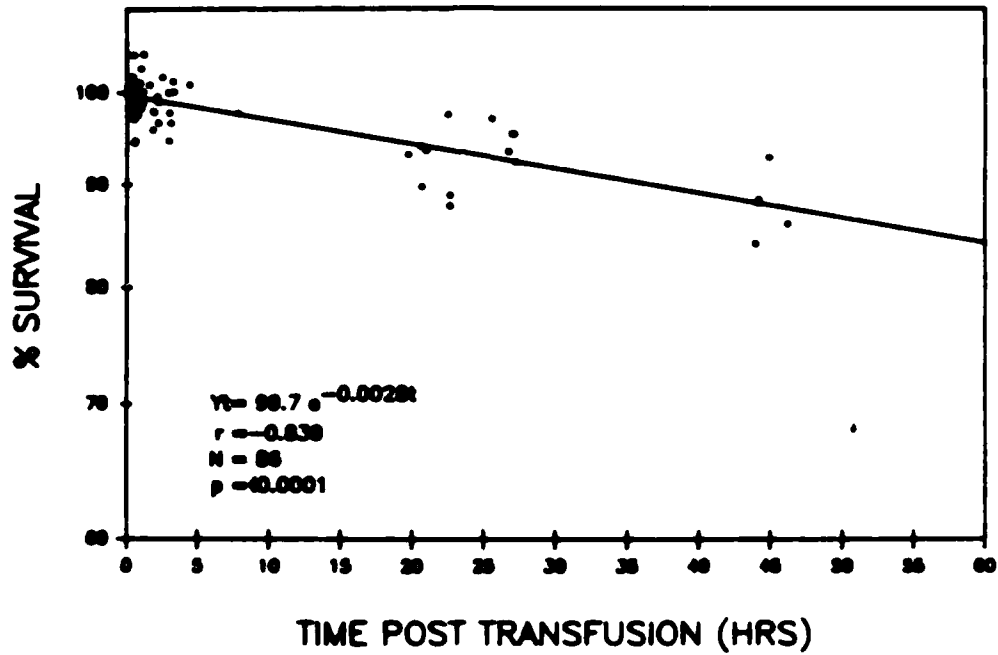
Figure 2.

Percent survival of unstored autologous rabbit RBC within the first 48 hours.

LONG TERM SURVIVAL OF UNSTORED RABBIT RBC

$Yt = 98.2 e^{-0.055t}$
$r = -0.941$
$N = 107$
$p = <0.00001$
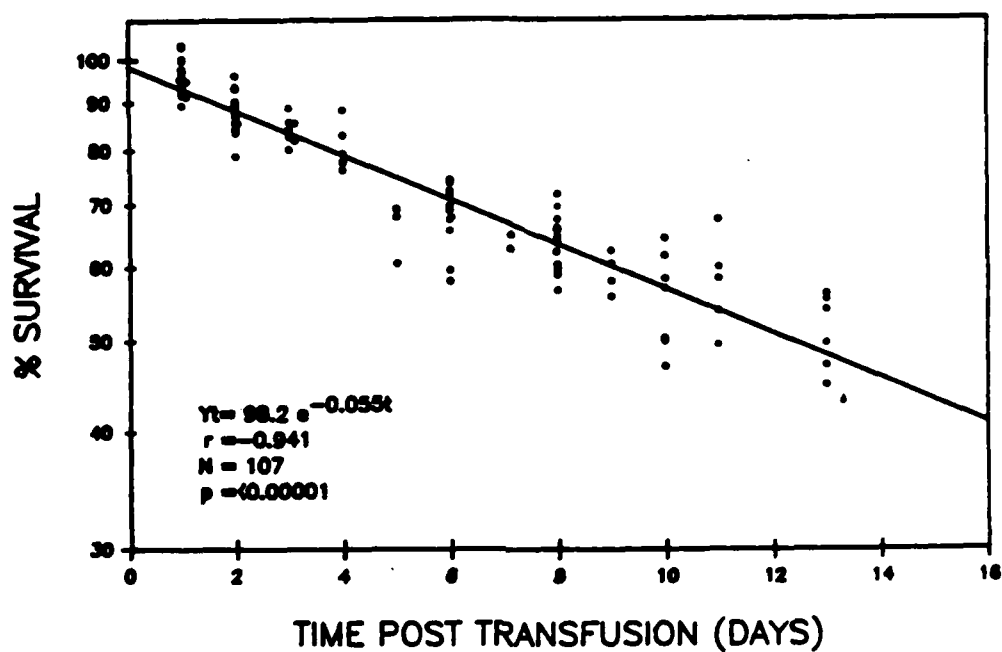
Figure 3

Percent survival of unstored autologous rabbit RBC between one and 13 days after transfusion.

SHORT TERM SURVIVAL OF CPD PRESERVED RABBIT RBC
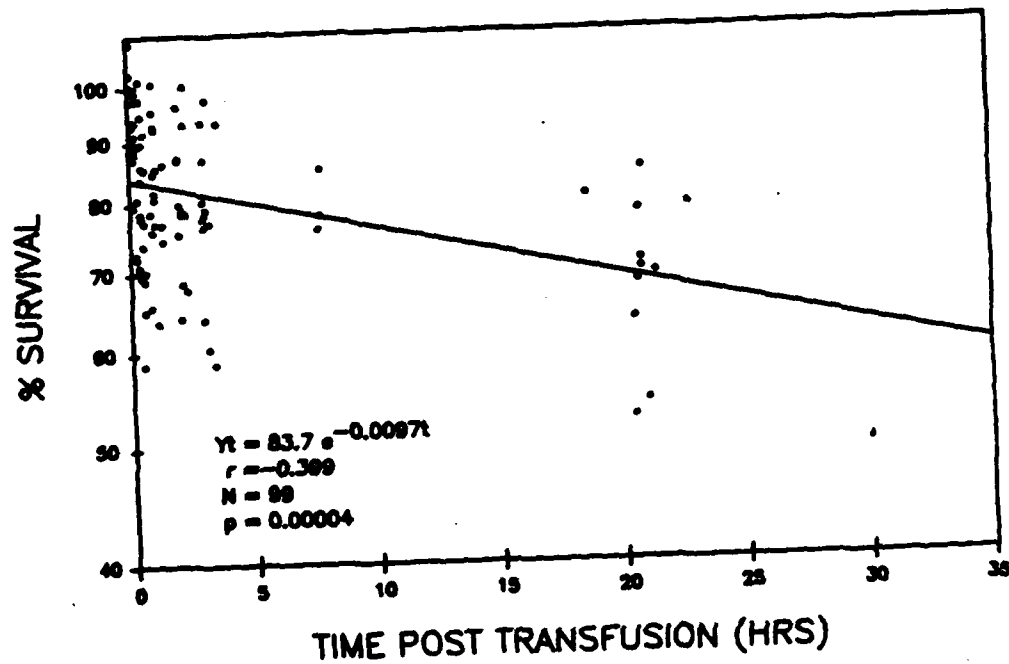
Figure 4

Percent survival of CPD stored rabbit RBC within the first 24 hours after transfusion. These data were obtained in a total of 21 animals.

Short term RBC survival studies of ADSOL RBC

The percent survival of ADSOL stored RBC correlated negatively with the time (r = -.776, n = 120, p < 0.00001). These data are plotted on Figure 5. The y-intercept was at 80.6% and the rate of destruction was 4% per hour and t 1/2 was 17.3 hours.

Short term RBC survival studies of RBC stored in C3 depleted CPD plasma

RBC survival regression during the first 24 hours after transfusion of RBC stored in C3 depleted CPD plasma is shown in Figure 6. There was a significant negative correlation between the time after transfusion and the percent RBC survival (r=-.448, n=156, p< 0.00001). The y-intercept was at 84.9 per cent and the rate of destruction was 1.8% per hour and the t 1/2 was 38.5 hours.

Analysis of Double vs Single Label Methods

In order to evaluate whether the survival of stored RBC can be measured correctly by a single label method, RBC survival determined by double label in 49 rabbits were re-analyzed using the Cr survival values obtained during the first one hour. The first sample was collected 6.34 ± 1.74 minutes after transfusion. Mean survival at 6.3 minutes was 90.1% ± 15.9, whereas the extrapolated y-intercept was 92.6% ± 15.4. Since extrapolated intercept was less than 100%, single label technic would overestimate RBC survival. This point is further illustrated by a study shown in Figure 7. This shows the rrate of early destruction in vivo of RBC stored in CPD for 50 days. In only one case the regression line of the early destruction could be extrapolated to 100% survival.

Long term RBC survival of stored RBC

Long term survival studies were carried out in 46 rabbits who received stored RBC. In six cases a clear collapse curve was observed. The survival data of the remaining cases were combined and plotted on semilogarithmic coordinates as a function of time. These data are shown in Figure 8. There was a significant negative correlation between the percent survival and the time after transfusion (r=-0.765, N=166, p<0.00001). The rate of destruction was 7.68 percent per day and the t 1/2 was 9.03 days.

The relationship between the length of storage and the individual t 1/2 values are plotted on Figure 9. There was a negative correlation between these variables (r = -0.402, n = 42, p < 0.01)

The 51-Cr content in various organs.

Normal Unstored RBC

Liver

On the average, 4.73 ± 1.41% of the total injected RBC-bound radioactivity and 5.03 ± 1.47% of the radioactivity in the surviving RBC was recovered

SHORT TERM SURVIVAL OF ADSOL PRESERVED RABBIT RBC
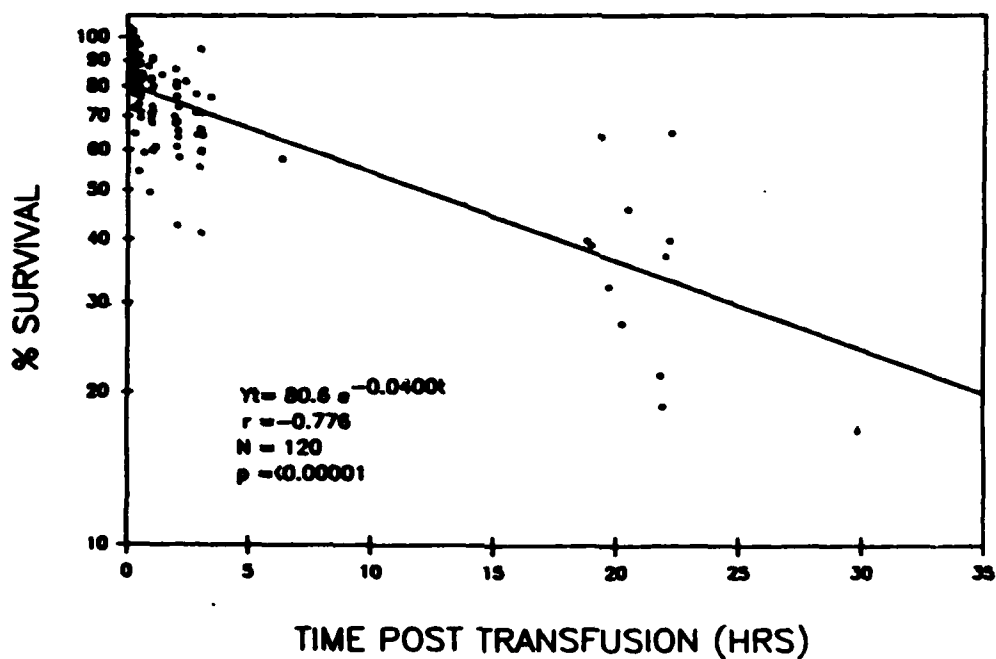
Figure 5

Percent survival of ADSOL stored rabbit RBC within the first 24 hours after transfusion. These data were obtained in a total of 26 rabbits.

SHORT TERM SURVIVAL OF RABBIT RBC STORED IN
C3 DEPLETED PLASMA

Y% = 84.9 e$^{-0.018t}$
r = -0.448
N = 156
p = <0.0001

TIME POST TRANSFUSION (HRS)

Figure 6

    Percent survival of rabbit RBC stored in C3 depleted CPD plasma within the first 24 hours after transfusion. These data were obtained in a total of 31 rabbits.

TRANSFUSION OF 50 DAY OLD CPD BLOOD TO THREE
RECIPIENT RABBITS
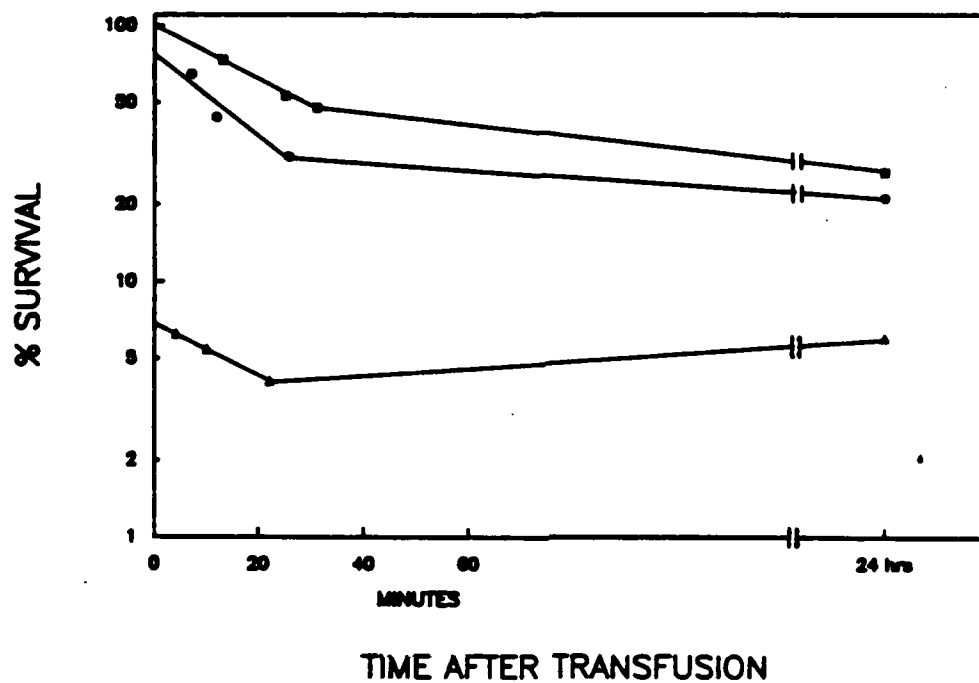
Figure 7

Destruction rates of RBC stored in CPD for 50 days in three rabbits.

LONG TERM SURVIVAL OF STORED RABBIT RBC

$$Yt = 97.7\ e^{-0.077t}$$
$$r = -0.765$$
$$N = 166$$
$$p = <0.00001$$

TIME POST TRANSFUSION (DAYS)
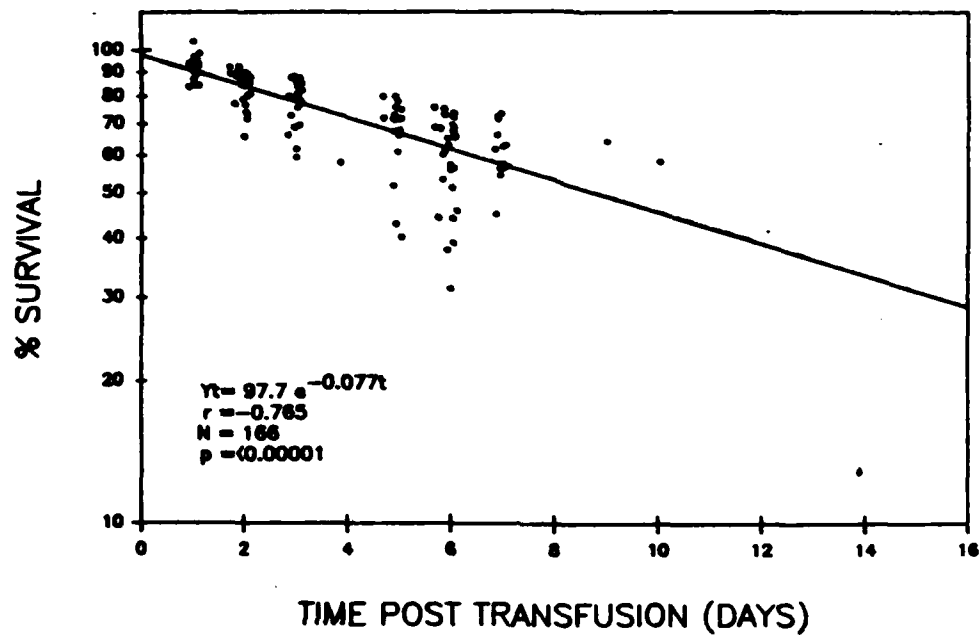
% SURVIVAL

Figure 8

Percent survival of stored RBC between one and 15 days after transfusion.

t 1/2 OF STORED RABBIT RBC
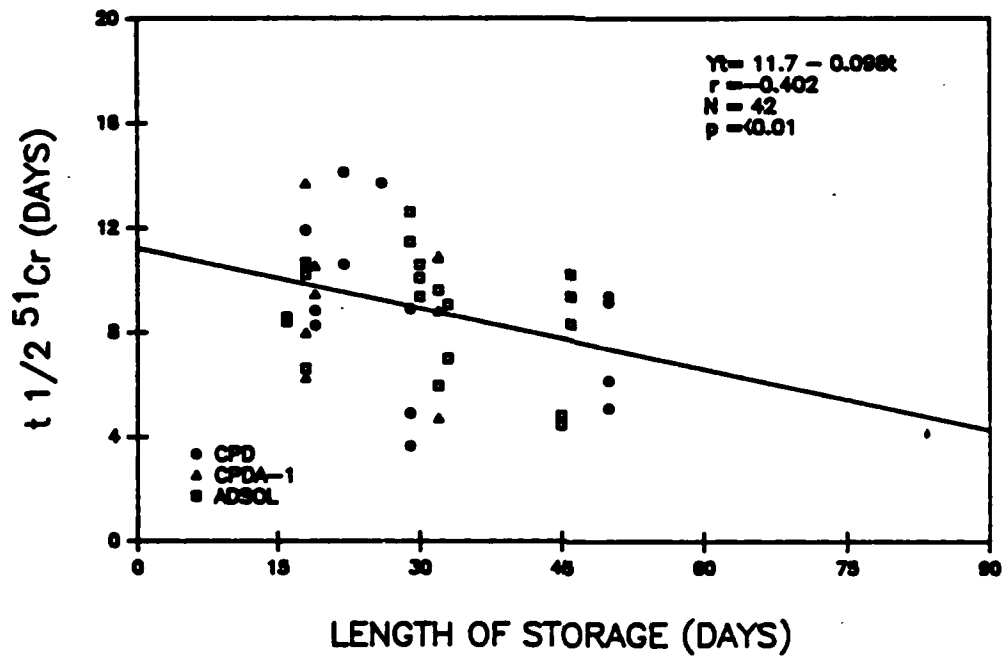
Figure 9

The relationship between t 1/2 Cr and the length of storage. Three different storage methods were used.

in the liver. These quantities correlated positively with the weight of the liver ($r = 0.562$ p = 0.005 and $r = 0.532$ p = 0.009, respectively), but not with the percent RBC survival (%S) or with the time after transfusion. On the average, $0.057 \pm 0.01\%$ of the surviving RBC were recovered per each gram of liver. These data indicate that the liver was not involved in the destruction of the senescent RBC present in the normal blood. The higher recovery of radioactivity in the larger livers indicated that they had proportionally more blood flow. Thus, the contribution of the circulating labeled RBC to the liver radioactivity (%RBCliv) could be expressed as follows:

$$\%RBC\ liv = 0.057*(liver\ weight,\ g.)*\%S.$$

Spleen

On the average, $0.319 \pm 0.188\%$ of the injected RBC-bound radioactivity was recovered in the spleen. This quantity correlated positively with the time after transfusion ($r=0.467$, $p=0.025$), negatively with the percent RBC survival ($r=-0.651$, $p=0.001$) and positively with the weight of the spleen ($r=0.668$, $p<0.001$). These data indicate that the spleen was involved in the destruction of the senescent, normal RBC and that the ability of the spleen to phagocytize was limited by its size. The percent RBC recovered per gram of spleen weight also correlated negatively with the percent RBC survival ($r = -0.893$, $p < 0.001$), as shown in Figure 10. The percent RBC recovered per gram of spleen correlated positively with the time after transfusion ($r = 0.782$ p = 0.00001) as shown in Figure 11. Therefore, the quantity of normal, senescent RBC phagocytized in the spleen (%phspl) after various times of transfusion was calculated using the following formula:

$$\%phspl= (0.202+ 0.0075*t)*Wtspl,$$

where t equals time after transfusion in hours and Wtspl equals the weight of spleen in grams.

Kidneys

On the average, $1.252 \pm 0.851\%$ of the injected RBC-bound radioactivity and $1.32 \pm 0.872\%$ of the radioactivity in the surviving RBC was recovered in the kidneys. This quantity did not correlate with the time after injection, percent RBC survival or with the weight of the kidney. These findings are compatible with the prediction that the kidney is not the site of RBC destruction. Therefore, it is apparent that the radioactivity recovered in this organ reflected the amount of blood flow in kidneys. Thus, the quantity of the surviving RBC in the kidneys (%RBCKid) was calculated using the following formula:

$$\%RBCKid= 1.328* \%S$$

Lungs

On the average, $1.106 \pm 0.310\%$ of the injected RBC-bound radioactivity and $1.17 \pm 0.293\%$ of the radioactivity in the surviving RBC was recovered in the lungs. The percent recovery of labeled RBC in lungs correlated positively with the percent RBC survival ($r = 0.544$, $p = 0.016$) and negatively with the time after transfusion ($r = -0.555$, $p = 0.013$), but

TRANSFUSION OF UNSTORED RABBIT RBC

$Yt = 3.372 + 0.0324t$
$r = -0.907$
$N = 23$
$p = <0.00001$

Y-axis: % RBC/g SPLEEN

X-axis: % SURVIVAL
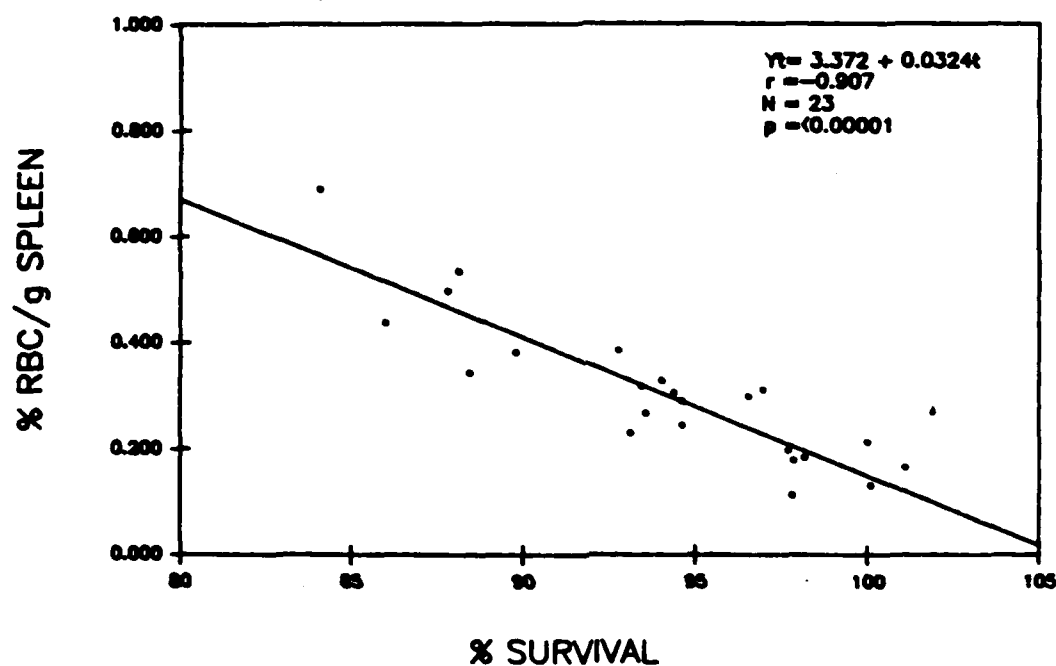
Figure 10

    The percent correlation between percent RBC survival and percent RBC recovered per gram of spleen. Unstored autologous RBC were transfused.

TRANSFUSION OF UNSTORED RABBIT RBC

Plot showing % RBC/g SPLEEN versus TIME POST TRANSFUSION (HRS). Fit line with:

$$Yt = 0.202 + 0.0075t$$
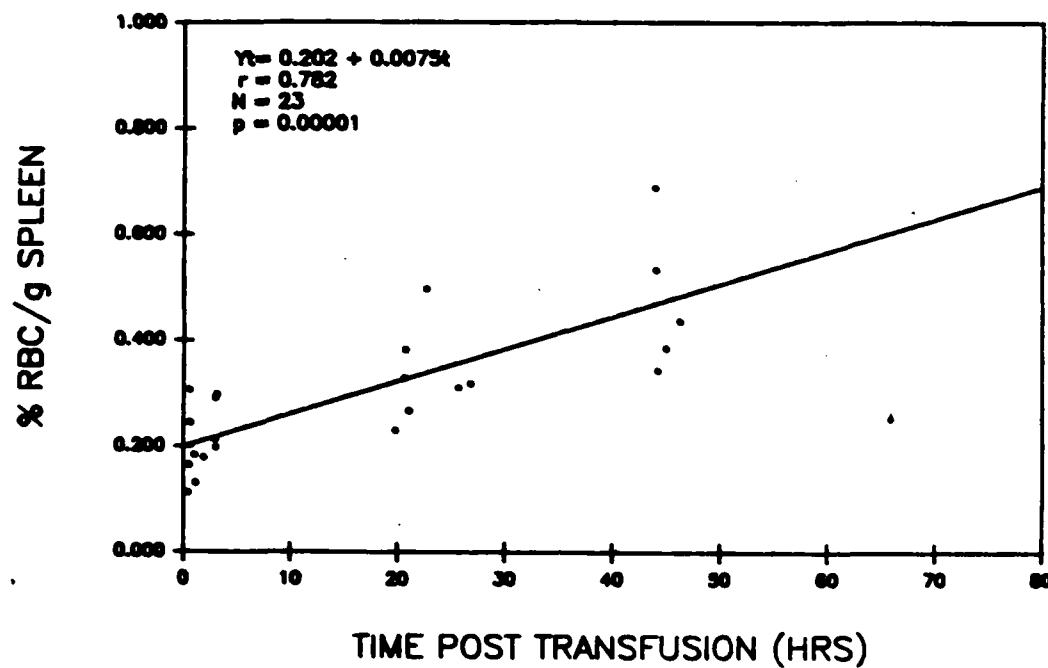$$r = 0.782$$
$$N = 23$$
$$p = 0.00001$$

Figure 11

The correlation between percent RBC recovered per gram of spleen and the time after transfusion.

not with the weight of the lungs. These data indicate that the amount of the radioactivity detected in the lungs reflected the amount of blood flow. It also appeared that the blood flow in lungs did not depend on the weight of the lungs. Therefore, the quantity of the surviving labeled RBC in the lungs (%RBClu) could be expressed with the following formula:

$$\%RBClu = 1.173 * \%S$$

Bone marrow

On the average, 3.563 ± 1.876% of the injected RBC-bound radioactivity was recovered in the bone marrow. The quantity of the total radioactivity in the bone marrow was estimated by multiplying the radioactivity in the marrow from one femur with 35.2. This quantity correlated positively with the time after transfusion ($r = 0.797$, $p < 0.0001$), negatively with the percent RBC survival ($r = -0.543$, $p = 0.016$) and positively with the weight of the bone marrow ($r = 0.781$, $p < 0.0001$). Both the total radioactivity and the radioactivity per gram bone marrow (expressed as percent of the injected RBC-bound radioactivity) are plotted as a function of the time after transfusion in Figures 12 and 13. Therefore, the percent of RBC sequestered in the bone marrow (%RBCbm) after transfusing fresh RBC could be expressed with the following formula:

$$\%RBCbm = (0.052 + 0.001 * t) * wtfem * 35.2,$$

where t equals the time in hours after the transfusion and wtfem equals the weight of the bone marrow in one femur.

Our data indicate that normal, senescent rabbit RBC were destroyed in both the bone marrow and the spleen. The sum of RBC recovered in bone marrow and spleen correlated positively with time after transfusion ($r = 0.801$ $p = 0.00004$; Figure 14) and it correlated negatively with the RBC survival ($r = 0.577$, $p = 0.0$; Figure 15). The sum of RBC recoveries in blood, bone marrow, and spleen was plotted as a function of time (Figure 16). A negative correlation was observed ($r = -0.618$, $p = 0.005$). On the average, 97.1% of the injected dose was accounted for at 24 hours.

Stored RBC.

Recovery of stored RBC in various organs

The post transfusion recoveries of stored RBC in lungs or kidneys (1.231 ± 0.366% and 1.280 ± 0.280% of the surviving RBC, respectively) were similar to those of autologous RBC, indicating that stored nonviable RBC were not phagocytized in these organs. However, the post transfusion recoveries of stored RBC in spleen, liver, and bone marrow were higher than after transfusion of fresh RBC. After subtracting the calculated normal background using formulas derived in the previous section, the degree of phagocytosis could be determined. The mean percentages of differently stored RBC surviving in blood or phagocytized by liver, spleen or bone marrow are shown in Table 1. For RBC stored by

TRANSFUSION OF UNSTORED RABBIT RBC

Plot with y-axis labeled "% RBC IN BONE MARROW" (0 to 10) and x-axis labeled "TIME POST TRANSFUSION (HRS)" (0 to 70). Inset text: Yt= 1.922 + 0.084t; r = 0.796; N = 19; p = 0.00004.
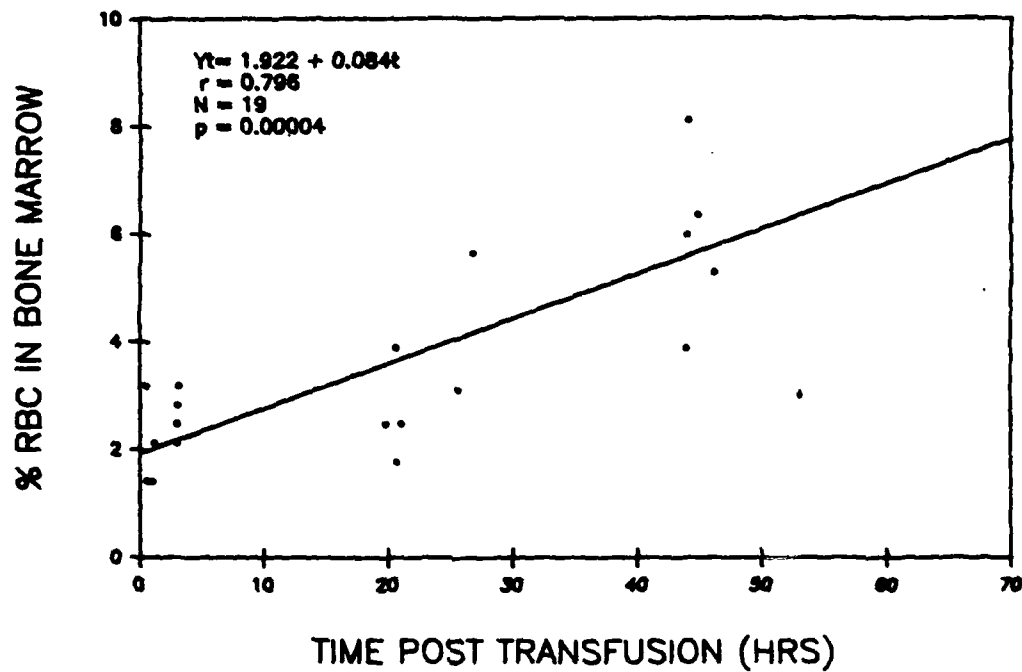
Figure 12

The recovery of autologous, unstored rabbit RBC in bone marrow after transfusion.

TRANSFUSION OF UNSTORED RABBIT RBC
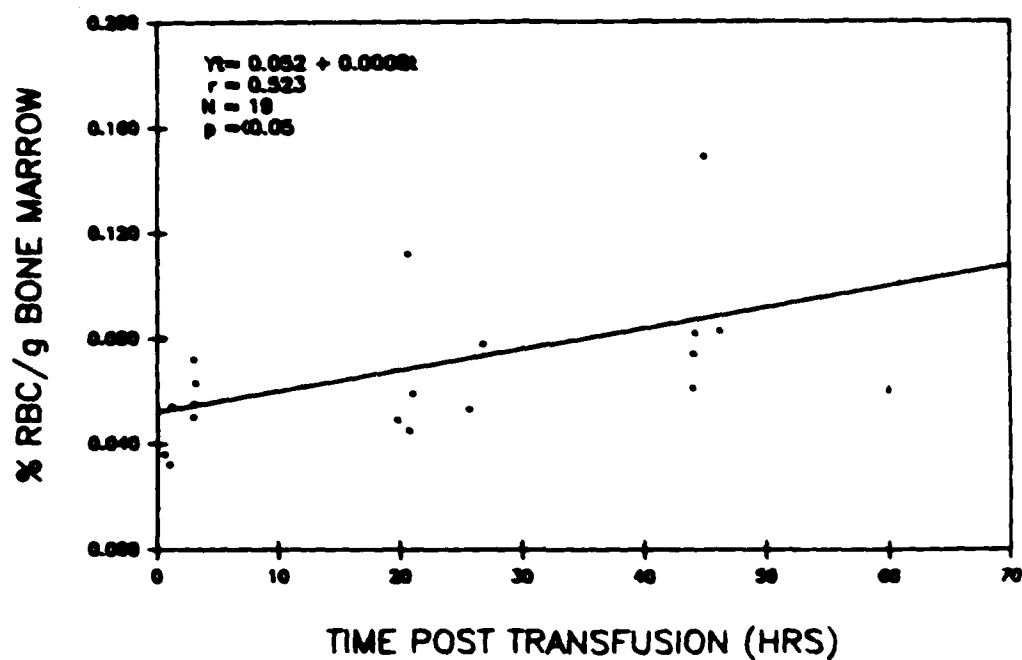
Figure 13

　　　　Percent　　autologous,　　unstored　RBC　recovered　in　bone　marrow　after transfusion.

TRANSFUSION OF UNSTORED RABBIT RBC

Yt= 2.17 + 0.088t
r = 0.801
N = 19
p = 0.00004
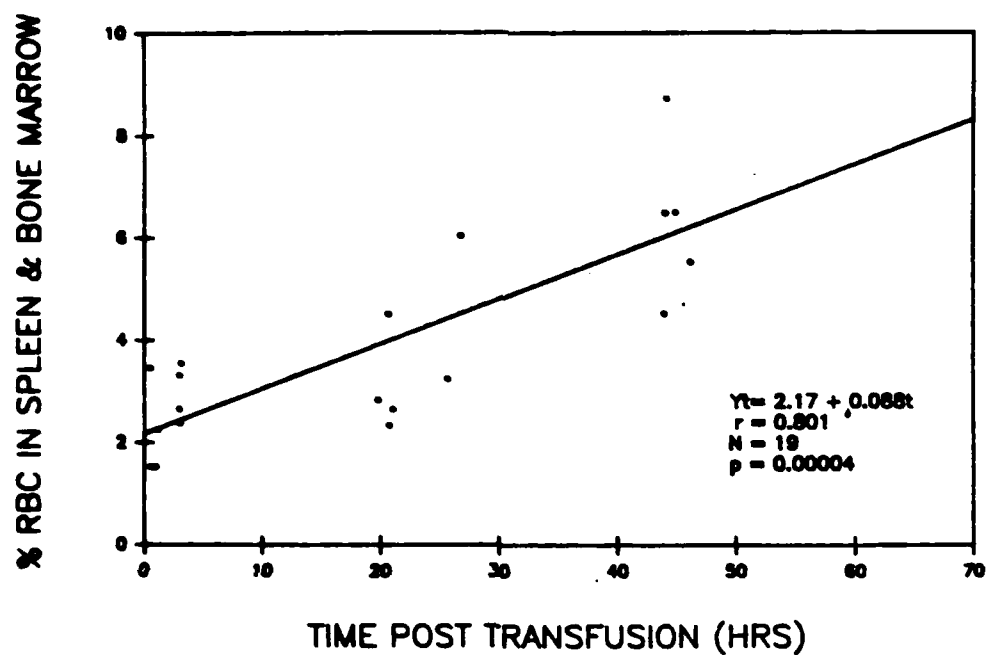
Figure 14

The sum of autologouus, unstored RBC recovered in spleen and bone marrow after transfusion.

TRANSFUSION OF UNSTORED RABBIT RBC
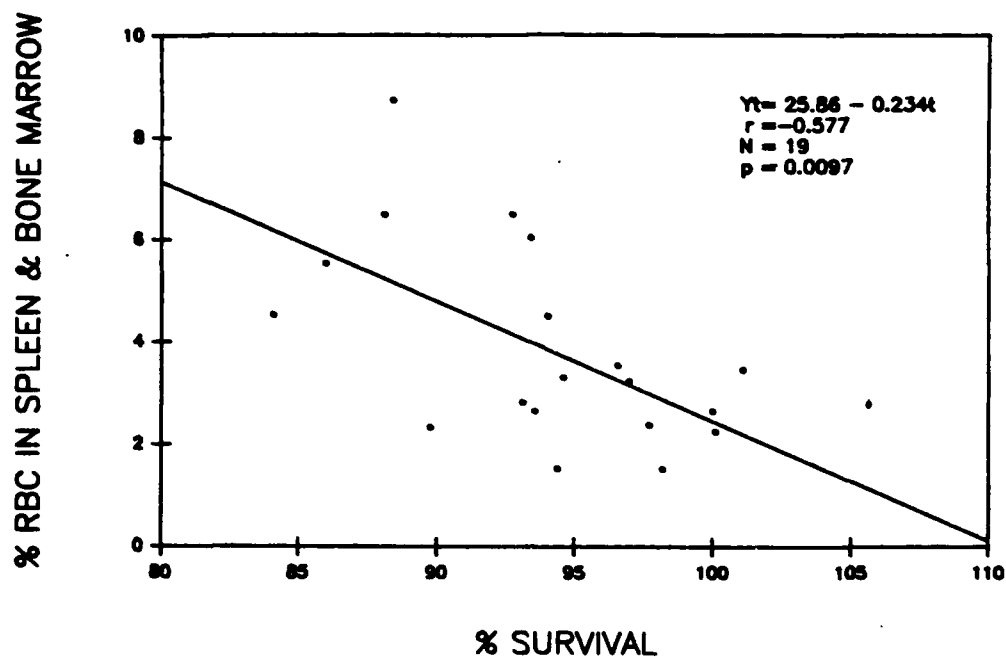
Figure 15

The correlation between percent survival of autologous unstored RBC and the sum of recovered labeled RBC in spleen and bone marrow.

TRANSFUSION OF UNSTORED RABBIT RBC
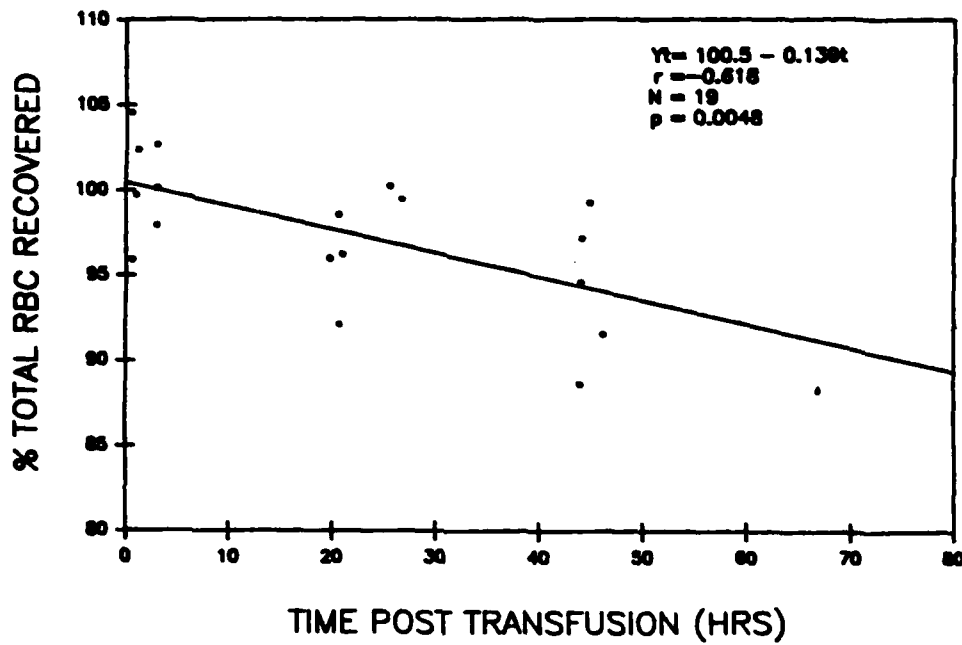
Figure 16

    The sum of recoveries of autologous unstored rabbit RBC in blood, bone marrow, and spleen after transfusion.

Table 1
The average percent survival of stored RBC in blood and percent of nonviable RBC phagocytized in liver, spleen, and bone marrow. Rabbit RBC had been stored at 4C in CPD, ADSOL, and in C3-depleted CPD for the stated length of time.

Storage Type	CPD	ADSOL	CPD (-C3)
Length of Storage (days)	19.0 ± 1.90 (N=21)	1.46 ± 10.66 (N=26)	18.87 ± 0.62 (N=31)
% Survival	72.0 ± 11.2 (N=21)	56.0 ± 21.6 (N=26)	72.2 ± 20.0 (N=31)
% Phagocytized in Liver	5.76 ± 4.94 (N=21)	7.48 ± 6.51 (N=26)	4.78 ± 6.17 (N=31)
% Phagocytized in Spleen	1.95 ± 1.65 (N=21)	2.39 ± 1.97 (N=26)	2.44 ± 1.785 (N=31)
% Phagocytized in BM	20.21 ± 12.73 (N=12)	37.33 ± 22.67 (N=15)	24.49 ± 18.1 (N=27)
Total Phagocytized	27.77 ± 13.78 (N=12)	46.76 ± 23.7 (N=15)	32.07 ± 23.16 (N=27)

various technics, the majority of nonviable RBC were phagocytized in the bone marrow. A larger fraction (20.7%) of nonviable CPD stored RBC were phagocytized in the liver than of ADSOL stored RBC (16.0%) or of RBC stored in C3 depleted CPD plasma. The correlation coefficients between the percent RBC survival and the percentage of RBC phagocytized in different organs are shown in Table 2. The RBC survival correlated negatively and most strongly with liver phagocytosis for CPD stored RBC, whereas for the other two preservation technics the strongest negative correlations were obtained between RBC survival and phagocytosis in the bone marrow.

Pearson correlation coefficients between the time after transfusion and the survival or organ phagocytosis of nonviable RBC of differently stored blood units is shown in Table 3. Regardless of the preservation method, the degree of phagocytosis by the liver did not change as a function of time after transfusion. The amount of RBC phagocytized by spleen increased with time regardless whether they had been stored in ADSOL or C3 depleted CPD.

DISCUSSION

This report contains data and analyses not given in the previous annual reports. These include a comprehensive analysis of survival data of fresh and stored rabbit RBC. We have also quantitated the phagocytosis of both senescent and nonviable stored RBC in various organs.

In addition, we show previously unreported data on rabbit red cell mass utilizing 51-Cr method. The mean red cell mass in male rabbits was 16.7 ± 2.01 ml per kg of body weight. These data are similar to those of Chalmers (9), but somewhat smaller than the mean value of 18.0 ± 2.0 ml obtained by Prince (10). We studied male rabbits, whereas Prince utilized nonpregnant female rabbits. We do not know whether the differences between the results could be attributed to differences in sex. Our study showed a strong positive correlation between the red cell mass and body weight of the rabbit, and a weak negative correlation between rabbit weight and ml of red cells per kg of body weight. It is known from human studies that red cell mass correlates better with lean body mass (body surface area) than with body weight (11). It is possible that also in rabbits the red cell mass per kg was less in larger (fatter) rabbits than in smaller (leaner) ones.

We compared survival data of stored homologous and fresh, autologous RBC. The survival curves of fresh RBC were analyzed in two groups of rabbits. In the first group, the time period of the study covered the first 48 hours after transfusion, whereas in the second group the time period extended from day one to day 13. The analysis of the survival was done in the traditional way, assuming an exponential clearance rate of the labeled RBC. The rate of destruction in the first group was only slightly faster (t 1/2 Cr 10.3 days) than in the second group (t 1/2 Cr 12.7 days). Most likely the apparent differences in the t 1/2 Cr resulted from the method of analysis, which considered only the exponential mode of destruction. In reality the 51-Cr survival regression is a composite of both linear and exponential clearance rates (12). These findings suggest

that the rate of destruction of fresh RBC is similar in both the early and late phases of the survival curve. Our results were similar, though slightly lower than those reported by others, who give t 1/2 values ranging from 13 to 18 days (13 - 15).

In contrast, stored RBC had two different rates of destruction, the early fast rate and a later slow rate. It was obvious that even the early rate of destruction had more than one component. Some RBC were apparantly removed immediately following transfusion since the y-intercepts of the survival curves were below 100% (Figures 4,5, and 6). The results showed that the majority of nonviable RBC in stored CPD blood were removed early on, with relatively little removal after the first few hours (Figure 4). However, the removal of ADSOL stored RBC continued for the first 24 hours after transfusion (Figure 5). These differences were even clearer in the studies where data were obtained only at the time the rabbit was sacrificed (Table 3). These data show that the survival of CPD stored RBC did not correlate with the time after transfusion, whereas the survival of RBC stored either in ADSOL or C3 depleted CPD plasma did.

Figure 5 shows that in several cases RBC that had been stored in C3 depleted CPD plasma (generated by injection of the donor rabbits with cobra venom factor (CVF) from either Indian or Egyptian cobra) had poor survival. Thus, it is obvious that in addition to deleting C3 from plasma CVF had other deleterious effects. Since it is known that CVF generates C5 convertase activity (16-17), it is possible that C5b6 complexes were present in donor plasma. These complexes can bind directly to bystander RBC (18) which lyse intravascularly upon infusion to the recipient rabbits by the mechanism of reactive hemolysis (19-21). Although reactive hemolysis is not a well known concept in immunohematology, the model described above could well be an example of such a phenomenon.

If RBC destruction occurs immediately during transfusion the single label technic would overestimate RBC survival. To avoid this problem it has been suggested that several blood samples should be collected after transfusion and the rate of the initial destruction determined (2). The y-intercept of these regressions would then reveal the true 100 % value. The data reported here do not support this approach. The mean recovery of stored RBC at the first sampling point (about 6 minutes after transfusion) was only about 90 %, whereas the mean recovery at the point of y-intercept was about 93 %. This point is illustrated in Figure 7. RBC that had been stored in CPD for 50 days were given to three recipient rabbits. In one case the intercept of the initial regression line could be extrapolated to 100 %, in other two cases, the y-intercepts were much below 100%. In all cases the 24 hour survivals were poor. Since the initial rate of clearance was different in the three rabbits, recipient factors might also govern the rate of removal of the nonviable RBC.

Inspection of long-term survival regression of stored RBC revealed that in six out of 24 studies a clear collapse curve could be discerned (22). Thus, in these cases there was evidence of sensitization to the homologous rabbit RBC. The remaining data were combined to study the fate of stored RBC in vivo after removal of nonviable RBC. These data revealed that on the average, t 1/2 Cr was 9.3 days (Figure 8), which is shorter than that of fresh RBC (Figure 3). However, visual inspection of these data reveals a fair amount of scatter. It appears that in many cases the

long term survival of stored RBC was in the normal range. These data confirmed the widely-held belief that stored RBC have a _potential_ for normal long term survival(1). When the individual t 1/2 values were plotted as a function of the storage length (Figure 9) the variability of the t 1/2 was evident. It appears that the longer the RBC had been stored the shorter t 1/2 values tended to be, although this was not always the case. It is possible that host factors as well as storage length influenced the t 1/2 of stored RBC.

The survival data support the recommendation made in our annual report: the most reliable information regarding RBC preservation is obtained by studies documenting normal long term survival. The y-intercept of such regression would most accurately represent the quantity of viable RBC in a unit of stored blood.

Since the nonviable stored RBC were cleared from circulation within 24 hours, studies to establish the site of their destruction were carried out during this time period. Phagocytosis in various organs was quantitated by measuring the total 51-Cr radioactivity in the organ after transfusing labeled stored RBC and subtracting the "normal background radioactivity" that would have been obtained after transfusing fresh autologous RBC. The recovery of 51-Cr labeled fresh autologous RBC in blood and in various organs was determined in 23 rabbits at various times during the first 48 hour period after transfusion. Mathematical formulas were developed to express the recovery of autologous RBC in various organs after transfusion. We measured the radioactivity in the bone marrow of one femur. This value was converted to total bone marrow uptake by multiplying it with 35.2. This factor was determined by 59-Fe ferrotkinetics studies which showed that on the average, the 59-Fe uptake by the bone marrow in the femur was 2.84% of the total 59-Fe uptake by bone marrow. Dietz has shown that the bone marrow content in the femurs, tibias and fibulae was 29% of total bone marrow (23). We also assumed that the quantity of phagocytosis by the various locations in bone marrow paralleled that of erythropoiesis. We felt that this method provided a reasonable estimate of total bone marrow phagocytosis. Utilizing this method we could account for almost all RBC-bound radioactivity given to the rabbit. The sum of recoveries of autologous fresh rabbit RBC in blood, bone marrow, and spleen at 24 hours after transfusion was 97.3%, indicating that about 3% of 51-Cr could have eluted. This estimate is ccompatible with previous reports (24).

RBC recovery in organs after transfusion of labeled fresh RBC represents either blood flow or phagocytosis of senescent RBC. To evaluate whether an organ was able to phagocytize senescent RBC, two criteria had to be met: 1) did the amount of radioactivity in the organ increase with the time after transfusion? and 2) did the amount of radioactivity in the organ correlate negatively with the percent RBC survival? If this was the case, the organ was considered to be a site of destruction of the senescent RBC.

The amount of fresh autologous RBC recoved in the spleen and bone marrow met both of these requirements. However, the amount of fresh autologous RBC recovered in the liver did not. Therefore we concluded that in the rabbit, only the bone marrow and spleen were involved in the destruction of the senescent RBC. It is remarkable that this information could be

gleaned from our data considering the fact that during the first 48 hours only a minor proportion of fresh autologous blood become senescent. Hughes Jones observed removal of senescent RBC during a longer period after transfusion and concluded that in the rabbit the senescent RBC are primarily removed by the bone marrow while the liver and spleen play only minor roles (24). Khonsari and Fudenberg on the other hand, report that senescent RBC are eliminated in the liver (25). However, the data by Khonsari and Fudenberg lack detail and therefore are difficult to understand. The differences between our data and those of Hughes Jones (24) could be explained in two different ways: either our technic of analyzing the liver radioactivity lacks precision or that the radioactivity detected in liver by Hughes Jones represents further breakdown of the hemoglobin beta chain in this organ.

The post transfusion recoveries of 51-Cr labeled fresh or stored RBC were similar in both lungs and kidneys. Thus it was obvious that these organs were not involved in the destruction of nonviable stored RBC. However, the post transfusion recoveries of stored RBC in liver, spleen and bone marrow were higher than those of fresh autologous RBC. Thus, these organs were involved in the destruction of stored nonviable RBC.

Table 1 shows the quantity of the nonviable RBC phagocytized by the bone marrow, liver and spleen after transfusing differently preserved RBC. Regardless of the preservation method, the majority (over 75%) of nonviable RBC were phagocytized by the bone marrow. The liver played a secondary role and even fewer nonviable RBC were phagocytized by the spleen. The fact that on the average about 100% of the injected RBC could be recovered in both the organs and blood lends credence to these calculations.

Table 2 shows the strength of negative correlation between the percent survival and the degree of phagocytosis by various organs for differently preserved RBC. Interestingly, for CPD stored RBC, phagocytosis by liver showed the strongest negative correlation with RBC survival.

Table 3 shows the relationship of RBC survival and phagocytosis by the various organs to the time after transfusion for differently preserved RBC. It is of interest that for CPD stored RBC the RBC survival did not decrease and the phagocytosis did not increase as a function of time. For any preservation technic, phagocytosis by the liver did not increase as a function of time. However, phagocytosis by both the spleen and bone marrow increased as a function of time.

These data indicate that phagocytes residing in the three reticuloendothelial organs function at different speeds. The phagocytes in the liver seem to be able to destroy RBC rapidly whereas those residing in the spleen seem to do so at a slower rate. The bone marrow seems to have both types of phagocytes.

In summary, we have presented new data and analysis of previously collected data in this report. These results show that the rabbit model is suitable for evaluating important issues in RBC preservation. We have also shown that the major site of destruction of the nonviable stored RBC is the bone marrow.

CONCLUSIONS

The results and analyses shown in this and previous reports indicate that the rabbit model is suitable for evaluating several RBC preservation related issues. The _in vivo_ behavior of stored RBC in rabbits is very similar to that in humans. The specific conclusions drawn from the currently reported studies are:

1. The size of RBC mass in rabbit correlates with rabbit weight and is 16.7 ± 2.01 ml per kg.

2. The rate of clearance of fresh normal rabbit RBC during the early period after transfusion (first 48 hours) is similar to that in the later period (between one and 13 days). The minor differences are most likely observed in the respective clearance rates due to the method of mathematical analysis of survival regressions. These analyses consider that RBC are cleared at a _single_ exponential rate.

3. Clearance of stored RBC after transfusion proceeded with two distinct rates; during the first 24 hours RBC were cleared rapidly whereas afterwards they were removed at a slower rate. The nonviable RBC were removed during the first 24-hour period as is the case in humans.

4. Nevertheless, even the early rate was a composite of more than one rate. This was obvious from the analysis of the RBC survival regressions during the first 24 hours after transfusion. The intercepts of the regression lines were below 100%.

5. Even when the RBC survival regressions were analyzed during the first one hour, the y-intercepts of the regression lines were below 100% (mean 92%). The differences between the 100% value and y-intercepts indicates the number of nonviable RBC removal during transfusion. Therefore, estimations of the quality of RBC preservation by single label method are not accurate.

6. On the average, long-term survival curves of stored RBC had a t 1/2 Cr of 9.03 days. This was shorter than the t 1/2 Cr of fresh RBC. However, in many individual cases the t 1/2 Cr was normal, indicating that the viable, stored RBC have a potential for long term survival. This is in accord with the data obtained in human studies. The short t 1/2 values could reflect host factors including disease or sensitization to RBC isoantigens.

7. When the time after transfusion increased, more labeled fresh RBC accumulated in the spleen and bone marrow, but not in the liver, implicating the spleen and bone marrow in the destruction of normal senescent RBC. This conclusion was supported by the finding that the percent survival of fresh RBC was negatively correlated with the recoveries of labeled RBC in the spleen and bone marrow.

Table 2

Correlation coefficients between the % RBC survival and % of nonviable RBC phagocytized in various organs following transfusion of CPD, C3 depleted CPD, and ADSOL stored RBC.

% Survival Correlated with Phagocytosis in	CPD Mean storage 19 days	ADSOL Mean storage 31.5 days	CPD (-C3) Mean storage 18.9 days
	(r; N; p)		
Liver	-.645; 21; 0.002	-.403; 26; 0.041	-.750; 31; <0.001
Spleen	-.389; 21; 0.08	-.488; 26; 0.011	-.804; 31; <0.001
Bone Marrow	-.578; 12; 0.048	-.711; 15; 0.003	-.895; 27; <0.001
Total	-.711; 12; 0.006	-.859; 15; <0.001	-.961; 27; <0.001

<u>Table 3</u>

Pearson correlation coefficients between the time after transfusion and percent survival of stored RBC and percent of nonviable RBC phagoccytized in various organs. Rabbit RBC had been stored in CPD, ADSOL and in C3 depleted CPD plasma.

Time after transfusion Correlated with	CPD	ADSOL (r; N; p)	CPD – C3
Survival	-.203; 21; NS	-.781; 26; <0.001	-.501; 31; p=.004
Liver Phagocytosis	-.031; 21; NS	.384; 26; NS	-.231; 31; NS
Spleen Phagocytosis	.104; 21; NS	.567; 26; p=.002	.522; 31; p=.003
Bone Marrow Phagocytosis	.076; 12; NS	.431; 15; NS	.500; 27; p=.008
Total Phagocytosis	.032; 12; NS	.603; 15; p=0.017	.478; 27; p=.012

8. Since only the bone marrow of one femur was sampled, the quantity of total bone marrow was obtained by multiplying the activity found in the femur with 35.2. This factor was derived from ferrokinetics studies revealing 2.84% deposition of the total bone marrow iron in a femur.

9. Utilizing the above described method to quantitate total bone marrow, we were able to calculate the total post-transfusion recovery of either fresh or stored RBC in the blood and organs of the rabbit. 97.3% of the total dose after transfusion of fresh RBC was recovered 24 hours later. This indicates about 3% elution rate of Cr, which is consistent with previous studies.

10. The post-transfusion recoveries of fresh and stored RBC in liver, spleen, kidney, lungs, and bone marrow were measured. Similar amounts of either fresh or stored RBC were recovered in the lungs as well as the kidneys, indicating that these organs were not involved in the elimination of stored RBC.

11. The post-transfusion recoveries of stored RBC in the liver, spleen, and bone marrow were higher than those of fresh RBC indicating that these organs were involved in elimination of stored RBC.

12. The quantity of stored RBC recovered in the three organs of the reticuloendothelial system was measured. It was shown that regardless of the preservation technic, the bone marrow was the major site of destruction of nonviable RBC, with the spleen playing quantitatively the least important role.

13. The correlations between the recoveries of variously stored RBC in the three reticuloendothelial organs and time after transfusion show that the RBC uptake by the liver did not increase as a function of time, whereas that by the spleen and bone marrow did. Our data suggest that on the basis of the rate of phagocytosis, at least two types of phagocytes are involved in removal of nonviable stored RBC. The liver contains rapidly phagocytizing cells, the spleen contains slowly phagocytizing cells and the bone marrow contains both types of phagocytes.

14. Removal of C3 from the plasma of donor rabbits by cobra venom factor treatment was used as a preservation method to prevent accumulation of C3 on the RBC membrane during storage at 4C. The survival of RBC stored in this fashion, however, was very variable. In some cases the survival was very good, in others it was very poor. It is suggested that "reactive hemolysis" was responsible for the rapid destruction of RBC in these cases.

RECOMMEDATIONS

1. The data presented show that the rabbit model can be used effectively to evaluate various issues involved in RBC preservation. Further use of this model for other studies is recommended.

2.   It is clear that even more information than is reported here can be gleaned from the body of data collected previously. However, such an analysis is time-consuming and is not currently possible due to lack of funding.

3.   Our results show, as do those of Hugh-Jones, that the main site of destruction of senescent rabbit RBC is the bone marrow. However Hughes-Jones found that some destruction took place also in the liver, whereas Khansari and Fundenberg maintain that senescent RBC are mainly destroyed in the liver. This discrepancy should be resolved. To that end, the technic of evaluating liver phagocytosis should be modified. For instance, it might be advisable to remove the circulating blood from the liver by saline injections through the portal vein before the organ is counted for radioactivity.

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